REMARKS

Reconsideration of this application is respectfully requested.

A complete listing of the claims in this application is provided above. The complete listing identifies claims that were previously presented, claims that were canceled, and two new claims.

Status of Claims After Entry

After entry of the claims identified in the complete listing above, the status of the claims will be as follows:

Amended claims: None.

Canceled claims: None

New claims added: 1795-1796.

Pending claims presented for further examination: 569-571, 573-575, 577, 582-589, 592-594, 597-600, 602-604, 607-608, 610-612, 614-624, 634-635, 637-638, 641-642, 646, 648-651, 656-661, 667, 670, 707-714, 716-717, 719-723, 725-727, 729, 734-747, 749-752, 754-756, 759-760, 762-764, 766-776, 786-787, 789-790, 793-794, 796-797, 800-803, 808-813, 819, 822, 859-866, 868-869, 871-875, 877-879, 881, 886-899, 901-904, 906-908, 911-912, 914-916, 918-928, 938-939, 941-942, 945-949, 952-955, 960-965, 971, 974, 1011-1018, 1020-1021, 1023-1027, 1029-1031, 1033, 1038-1051, 1053-1056, 1058-1060, 1063-1064, 1066-1068, 1070-1080, 1090-1091, 1093-1094, 1097-1099, 1101, 1104-1107, 1112-1117, 1123, 1126, 1163-1170, 1172-1173, 1175-1179, 1181-1183, 1185, 1190-1200, 1204, 1208-1209, 1212-1216, 1218-1244, 1248-1249, 1253, 1255-1258, 1263-1270, 1272, 1275, 1278-1294, 1296-1328, 1331-1332, 1334-1351, 1353-1354, 1357-1358, 1360, 1362-1369, 1372-1380, 1383, 1386-1391, 1393-1407, 1409-1487, 1490-1491, 1493-1516, 1518, 1520-1525, 1527, 1530-1539, 1541, 1544-1568, 1570-1585, 1587, 1592-1612, 1614-1615, 1618-1621, 1623-1628, 1631-1632, 1635-1647, 1649-1656, 1658, 1660-1667, 1670-1677, 1679-1680, 1682, 1685-1773 and 1775-1796.

Changes to the Claims

New claims 1795-1796 have been added above. Both claims are directed to a process for determining the sequence of a nucleic acid of interest. Claim 1795 comprises four steps. The first step recites "providing or generating detectable non-radioactively labeled nucleic acid fragments comprising: (a) a sequence complementary to said nucleic acid of interest or a portion thereof, and (b) fluorescent labels covalently attached, directly or through a linkage group, to said fragments." The second step calls for "subjecting said labeled fragments to a sequencing gel to separate or resolve said labeled fragments." The last two steps of claim 1795 recite "detecting non-radioactively said separated or resolved fragments by means of said attached fluorescent labels; and determining the sequence of said nucleic acid of interest from said detected fragments."

Like claim 1795, claim 1796 recites four steps. The first step recites "providing or generating detectable non-radioactively labeled nucleic acid fragments comprising: (a) a sequence complementary to said nucleic acid of interest or a portion thereof, and (b) different fluorescent labels covalently attached, directly or through a linkage group, to said fragments." The second step calls for "subjecting said labeled fragments to a sequencing gel to separate or resolve said labeled fragments." The last two steps provide for "detecting non-radioactively said separated or resolved fragments by means of said attached different fluorescent labels; and determining the sequence of said nucleic acid of interest from said detected fragments."

It is believed that the subject matter of new claims 1795 and 1796 is fully supported by the original specification. With respect to support for the fluorescent elements recited in both new claims, Applicants point to the table below:

New Claim No.	Recitation in New Claim	Description/Support in the Specification
1795	fluorescent labels	Page 47, line 11 ("second fluorescent dye") Page 47, lines 12-14 ("several sets of fluorescent labels attached to the cellular DNA") original claims 42 and 130 ("said Sig chemical moiety includes or comprises a fluorescing component")
1796	different fluorescent labels	Page 47, 1st ¶ ("By allowing one set of labeled clones to hybridize to the chromosomes and then adding a fluorescent stain to the label, the set of clones and their locations can be visualized and will flouresce[sic] with a particular color. A second set of labeled clones could then be used and reacted with a second fluorescent dye. The same process can be repeated a number of times. Thus one can, if desired, have several sets of fluorescent labels attached to the cellular DNA at different but specific locations on each of the chromosomes.") Page 48, 1st ¶("If necessary, two sets of labels can be used-- one which would be specific for chromosome 23 and one for some other chromosome. By measuring in each cell the ratio of the two labels, which might be of different colors, it is possible to identify the cells which show an abnormal number of chromosome number 23.")

Entry of new claims 1795 and 1796 is respectfully requested.

Supplemental Declaration Of Dr. James J. Donegan

In addition to the presentation of new claims 1795 and 1796, Applicants are submitting herewith attached as Exhibit 1 the Supplemental Declaration of Dr. James J. Donegan.[1] It is believed that Dr. Donegan's Supplemental Declaration, offered by a person skilled in the art, further establishes the following:

- that the '069 specification discloses fluorescent labels as recited in claim 1795.
- that the '069 specification also discloses different fluorescent labels as recited in claim 1796.
- that fluorescent labels are synonymous with fluorophores.
- that different fluorescent labels exhibits different spectral characteristics.

Applicants respectfully request that consideration be given to Dr. Donegan's Supplemental Declaration as it might reflect on the patentability of any pending or newly-submitted claims, and/or the appropriateness of their request for interference.

Submission of New Request for Interference

Applicants' attorneys are preparing a new request for interference that will be submitted shortly. Their new request will take into account the new rules of 37 C.F.R. § § 41.200-208, which took effect September 13, 2004.

[1] As indicated on the first page of Dr. Donegan's Supplemental Declaration (Exhibit 1), he is the same person who submitted a Declaration in this application in July 1998.

SUMMARY AND CONCLUSIONS

A complete listing of the claims effected by this paper is provided above. Two new claims, 1795 and 1796, have been added. No other changes to the claims have been made.

Presented for further prosecution on the merits are the following claims: 569-571, 573-575, 577, 582-589, 592-594, 597-600, 602-604, 607-608, 610-612, 614-624, 634-635, 637-638, 641-642, 646, 648-651, 656-661, 667, 670, 707-714, 716-717, 719-723, 725-727, 729, 734-747, 749-752, 754-756, 759-760, 762-764, 766-776, 786-787, 789-790, 793-794, 796-797, 800-803, 808-813, 819, 822, 859-866, 868-869, 871-875, 877-879, 881, 886-899, 901-904, 906-908, 911-912, 914-916, 918-928, 938-939, 941-942, 945-949, 952-955, 960-965, 971, 974, 1011-1018, 1020-1021, 1023-1027, 1029-1031, 1033, 1038-1051, 1053-1056, 1058-1060, 1063-1064, 1066-1068, 1070-1080, 1090-1091, 1093-1094, 1097-1099, 1101, 1104-1107, 1112-1117, 1123, 1126, 1163-1170, 1172-1173, 1175-1179, 1181-1183, 1185, 1190-1200, 1204, 1208-1209, 1212-1216, 1218-1244, 1248-1249, 1253, 1255-1258, 1263-1270, 1272, 1275, 1278-1294, 1296-1328, 1331-1332, 1334-1351, 1353-1354, 1357-1358, 1360, 1362-1369, 1372-1380, 1383, 1386-1391, 1393-1407, 1409-1487, 1490-1491, 1493-1516, 1518, 1520-1525, 1527, 1530-1539, 1541, 1544-1568, 1570-1585, 1587, 1592-1612, 1614-1615, 1618-1621, 1623-1628, 1631-1632, 1635-1647, 1649-1656, 1658, 1660-1667, 1670-1677, 1679-1680, 1682, 1685-1773 and 1775-1796.

Applicants believe that no additional fees are due in connection with this paper or the Supplemental Declaration being submitted herewith. Even with the addition of these two new claims, the total number of claims pending in this application is less than the number of previously paid for claims. In the event that any additional fees are due, however, Applicants hereby requests that the Patent and Trademark Office charge the amount of any such fees to Deposit Account No. 05-1135.

If a telephone conversation would further prosecution of the application, the Examiner is welcome to call Applicant's undersigned attorney at the number below.

Respectfully submitted,

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Dean Engelhardt et al.

Serial No.: 08/486,069

Filed: June 7, 1995

For: **NUCLEIC ACID SEQUENCING PROCESSES USING MODIFIED NUCLEOTIDES OR NUCLEOTIDE ANALOGS, AND OTHER PROCESSES FOR NUCLEIC ACID DETECTION AND CHROMOSOMAL CHARACTERIZATION USING SUCH MODIFIED NUCLEOTIDES OR NUCLEOTIDE ANALOGS** (As Previously Amended)

Group Art Unit: 1631

Ex'r: Ardin H. Marschel, Ph.D.

60 Executive Boulevard
Farmingdale, NY 11735-4716

Commissioner for Patents
Washington, D.C. 20231

SUPPLEMENTAL DECLARATION OF DR. JAMES J. DONEGAN

I, James J. Donegan, hereby declare as follows:

1. I am the same Dr. James J. Donegan who submitted a Declaration in the above-identified application in July 1998. My professional background, education, training and experience are as described in my *curriculum vitae* (cv) attached as Exhibit 1 to my July 1998 Declaration. A recent cv is attached, however, as Exhibit A to this Supplemental Declaration.

2. Enzo Life Sciences, Inc. has requested that I review as its senior scientist significant portions of the most recent prosecution history of United States Patent Application Serial No. 08/486,069, filed on June 7, 1995 ("the '069 application") in the name of Dean L. Engelhardt, *et al.* as inventors. The title of the Engelhardt application is "Nucleic Acid Sequencing Processes Using Modified Nucleotides or Nucleotide Analogs, And Other Processes For Nucleic Acid Detection And Chromosomal Characterization Using Such Modified Nucleotides or Nucleotide Analogs." Included for this particular review were the following documents:

- The Office Action mailed on July 1, 2003;
- Three of Applicants' responses:
    - December 21, 2001 Request For Interference Pursuant To 37 C.F.R. §607;
    - December 31, 2003 Amendment [Following Office Action of July 1, 2003];
    - July 13, 2004 Supplemental Amendment To Applicants' December 31, 2003 Amendment;
- A set of claims that were presented in Applicants' July 13, 2004 Supplemental Amendment;[1]
- Two new claims 1795 and 1796 which are being submitted to the U.S. Patent Office in this application;[2] and

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[1] **The set of claims include: 569-571, 573-575, 577, 582-589, 592-594, 597-600, 602-604, 607-608, 610-612, 614-624, 634-635, 637-638, 641-642, 646, 648-651, 656-661, 667, 670, 707-714, 716-717, 719-723, 725-727, 729, 734-747, 749-752, 754-756, 759-760, 762-764, 766-776, 786-787, 789-790, 793-794, 796-797, 800-803, 808-813, 819, 822, 859-866, 868-869, 871-875, 877-879, 881, 886-899, 901-904, 906-908, 911-912, 914-916, 918-928, 938-939, 941-942, 945-949, 952-955, 960-965, 971, 974, 1011-1018, 1020-1021, 1023-1027, 1029-1031, 1033, 1038-1051, 1053-1056, 1058-1060, 1063-1064, 1066-1068, 1070-1080, 1090-1091, 1093-1094, 1097-1099, 1101, 1104-1107, 1112-1117, 1123, 1126, 1163-1170, 1172-1173, 1175-1179, 1181-1183, 1185, 1190-1200, 1204, 1208-1209, 1212-1216, 1218-1244, 1248-1249, 1253, 1255-1258, 1263-1270, 1272, 1275, 1278-1294, 1296-1328, 1331-1332, 1334-1351, 1353-1354, 1357-1358, 1360, 1362-1369, 1372-1380, 1383, 1386-1391, 1393-1407, 1409-1487, 1490-1491, 1493-1516, 1518, 1520-1525, 1527, 1530-1539, 1541, 1544-1568, 1570-1585, 1587, 1592-1612, 1614-1615, 1618-1621, 1623-1628, 1631-1632, 1635-1647, 1649-1656, 1658, 1660-1667, 1670-1677, 1679-1680, 1682, 1685-1773 and 1775-1794 (copy not provided).**

[2] **Attached as Exhibit B.**

- The patent specification filed on June 7, 1995 (but claiming priority to June 23, 1982) [hereinafter "the '069 specification"].

3. Based upon my review of the pending claims, including the two new claims (1795 and 1796) being submitted to the U.S. Patent Office (Exhibit B), I understand that the invention in the '069 application is directed to nucleic acid sequencing processes and other processes for nucleic acid detection using modified nucleotides or modified nucleotide analogs. New claims 1795 and 1796 are processes directed to determining the sequence of a nucleic acid of interest. Both claims recite four steps. Claim 1795 recites a first step of providing or generating detectable non-radioactively labeled nucleic acid fragments comprising: (a) a sequence complementary to the nucleic acid of interest or a portion thereof, and (b) fluorescent labels covalently attached, directly or through a linkage group, to the fragments. The remaining three steps in claim 1795 call for subjecting the labeled fragments to a sequencing gel to separate or resolve the labeled fragments; detecting non-radioactively the separated or resolved fragments by means of the attached fluorescent labels; and determining the sequence of the nucleic acid of interest from the detected fragments. Claim 1796 also recites a first step of providing or generating detectable non-radioactively labeled nucleic acid fragments comprising: (a) a sequence complementary to the nucleic acid of interest or a portion thereof, and (b) different fluorescent labels covalently attached, directly or through a linkage group, to the fragments. The last three steps in claim 1796 call for subjecting the labeled fragments to a sequencing gel to separate or resolve the labeled fragments; detecting non-radioactively the separated or resolved fragments by means of the attached different fluorescent labels; and determining the sequence of the nucleic acid of interest from the detected fragments.

4. I have read the July 1, 2003 Office Action in which the Patent Examiner denied Applicants' Request For Interference filed on December 21, 2001.[3] I understand that the Patent Examiner has raised questions regarding claim language for "chromophore or fluorophore," "different indicator molecules," and "spectral characteristics."

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[3] REQUEST FOR INTERFERENCE

The Request for Interference, filed 12/21/01, is again acknowledged as having been received and is further responded to as follows. The Request for Interference is DENIED under 37 CFR§1.607 as being not fully supportive of instituting an interference regarding U.S. Patent 5,821,058 as requested. Consideration of the instant claims as well as the claims of said Patent reveals that either a "chromophore or fluorophore" or "chromophores or fluorophores" is required for detection practice in all of the claims of said Patent. In contrast, the instant claims discussed in said Request for Interference describe detection via non-radioactive labeling of nucleic acid fragments. The Request for Interference has not specifically discussed or supplied evidence that either "chromophore or fluorophore" or "chromophores or fluorophores" as detection practices are encompassed within the instant claims as interfering subject matter. The instant "non-radioactive" labeling citations in the instant claims are very generic and broad. Potentially persuasive arguments and/or evidence may be utilized in order to support the "chromophore or fluorophore" or "chromophores or fluorophores" practice of said Patent as being obvious species within the instantly claimed generic and broad "non-radioactive" labeling practice. Such argument(s) and/or evidence, however, has not been set forth in said Request for Interference.

Yet another limitation that is present in the claims of said Patent, but not sufficiently supported as being included as being interfering subject matter is that of the practice of utilizing "different indicator molecules" in the claimed methods. This is noted below as being NEW MATTER in the instant claims and thus also is a distinction between the claims of said Patent and the instantly claimed subject matter as not being supported as filed in the instant disclosure. This NEW MATTER issue also results in the differential labeling of fragments to differentially detect bases A, C, G, or T to lack interfering subject matter with the instant claims and is therefore insufficiently supported regarding the request for Interference. A related insufficiently supported limitation in the instant claims which is set forth in the claims of said Patent is that of "spectral characteristics" as being utilized in the practice of distinguishing nucleic acid fragments, such as on a sequencing gel. The instant claims lack any description of "spectral characteristics" much less their use per se for distinguishing nucleic acid fragments.

Therefore, for the reasons described above, said Patent is presently considered a non-obvious improvement specie of invention over the instantly claimed invention and therefore not properly subject to an Interference proceeding. To repeat from above, the Request for Interference, filed 12/21/01, is DENIED.

5. As Enzo's senior scientist, I am making this Supplemental Declaration to show that the '069 specification supports the subject matter for new claims 1795 and 1796, and in particular, support for the language "fluorescent labels" and "different fluorescent labels." I am also making this Supplemental Declaration to show that the term "fluorescent labels" is synonymous with the terms "chromophores or fluorophores." Furthermore, I wish to show by this paper that the term "different fluorescent labels" is synonymous with labels having different spectral characteristics. I have been told that my Supplemental Declaration will be submitted to the U.S. Patent Office as part of a Second Supplemental Amendment to the July 1, 2003 Office Action.

6. As set forth in my previously submitted cv, I am a scientist who is quite familiar with a number of techniques in biotechnology, including cloning and vector technology, nucleic acid amplification, nucleic acid detection and nucleic acid sequencing, to name a prominent few. Among my responsibilities at Enzo Life Sciences, Inc. have been the development of new nucleic acid amplification methods, new nucleic acid probe development based upon pathogenic agents, and new methods for non-radioactive nucleic acid detection.

7. In order to develop the new methods and agents referenced in the preceding paragraph, I have relied significantly on my education, background, training and experience in nucleic acid labeling, formatting and detection. During my career I was employed at various companies which have focused upon different labeling techniques. For instance, while at Genprobe, Inc. (San Diego, CA) from 1992-1993, I worked with nucleic acid probes labeled with acridinium esters on an abasic linker in a detection format that Genprobe calls the "Hybridization Protection Assay." In another instance, while at Syngene, Inc. (San Diego, CA) from 1991-1992, I performed assays with enzymes directly linked to oligonucleotide probes. At various times while at Enzo, I have developed and used nucleic acid probes in

which a labeling and signaling system is provided by a combination of biotin and streptavidin. While at Enzo I have also worked with oligonucleotides synthesized with two different fluorophores for the purpose of developing a FRET (Fluorescence Resonant Energy Transfer) assay.

8. Based upon my education, training, background and experience, I believe that at the time the first parent application of the now pending '069 application was filed in June 1982, the relevant art to the subject matter of nucleic acid sequencing, including new claims 1795 and 1796, would have included many, if not most of the areas, in which I had worked over several years. These areas include: nucleic acid detection, nucleic acid sequencing and the modification and labeling of nucleic acids for use in sequencing and detection processes. I consider myself to possess the level of skill, knowledge, training and experience of at least a person skilled in the art to which the present invention, including new claims 1795 and 1796, pertains.

9. It has been explained to me that a patent specification describes the subject matter of a claim, if the specification conveys, with reasonable clarity to a person skilled in the art, that the inventors were in possession of the subject matter recited in that claim. It has also been explained to me that to satisfy the written description requirement, the inventors do not have to utilize any particular form of disclosure to describe the subject matter of the claim under consideration. For instance, the description of the invention being claimed may be found in the working examples, in a more general description of the invention, or even in a combination of the examples and the general description.

10. As a person skilled in the art, it is my opinion and conclusion that the '069 specification reasonably conveys that at the time their application was first filed in June 1982, Applicants were in possession of the claimed invention directed to the

subject matter of new claims 1795 and 1796, including the features in which fluorescent labels and different fluorescent labels are covalently attached to nucleic acids for use in various processes, including nucleic acid sequencing. For reasons which are given below, I believe that the '069 specification reasonably conveys that the covalent attachment of fluorescent labels to nucleic acids is synonymous with the attachment of fluorophores to nucleic acids. Furthermore, the covalent attachment of different fluorescent labels to nucleic acids is synonymous with the attachment to nucleic acids of fluorophores which are distinguishable by their spectral characteristics.

THE '069 SPECIFICATION DISCLOSES THE FLUORESCENT LABELS RECITED IN CLAIM 1795.

11. As indicated in Paragraph 3 above, claim 1795 recites a first step of providing or generating detectable non-radioactive labeled nucleic acid fragments. The second feature of these fragments (b) recites "fluorescent labels covalently attached, directly or through a linkage group, to said fragments." In Example 9,[4] the '069 specification discloses fluorescent labels.

A (i) Fluorescent labels are clearly disclosed in Example 9.

Section I of Example 9[5] discloses fluorescent labels:

> I. Karyotyping
> (a) Select from a human gene library some 100 to 200 clones. Label them as described above, and for each clone locate its place or places of hybridization visually or with a low-light-level video system. For those clones which correspond to a unique sequence gene this determines the location of the cloned DNA on a particular human chromosome. Obtain several clones for each chromosome. Each of these labeled clones can be used to identify particular chromosomes. They can also be used in combination to identify each of the 46 chromosomes as being one of the 22 autosomal pairs or the X or the Y. By allowing ***one set of labeled clones*** to hybridize to the

[4] '069 specification, page 46, last paragraph, through page 48, first paragraph.
[5] '069 specification, page 46, last paragraph, through page 47, first paragraph.

> chromosomes and then adding a fluorescent stain to the label, the set of clones and their locations can be visualized and will ***flouresce with a particular color***. A ***second set of labeled clones*** could then be used and ***reacted with a second fluorescent dye***. The same process can be repeated a number of times. Thus one can, if desired, have ***several sets of fluorescent labels attached to the cellular DNA*** at different but specific locations on each of the chromosomes. These labels could be used for visual or computerized automatic karyotyping.
>
> [emphasis added]

As a person skilled in the art, it is clear to me that the above-quoted passage is describing fluorescent labels for attachment to nucleic acids, as recited in claim 1795.

A (ii) Fluorescent labels are also clearly disclosed in several originally filed claims.

In my review of the '069 specification, I note that two of the original claims recite language for fluorescing components while six other original claims recite three species of fluorescing components: fluorescein, rhodamine and dansyl. The original claims which recite "fluorescing component," "fluorescein," "rhodamine" and "dansyl" are set forth below (with emphasis added).

> ***(a) Original claim 42***
>
> 42. A nucleotide in accordance with Claim 1[6] wherein said Sig chemical moiety includes or comprises a ***fluorescing component***[7] attached thereto.

---

[6] **Original claim 1 recites:**

> **1. A nucleotide having the general formula P-S-B-Sig wherein P is the phosphoric acid moiety, S the sugar or monosaccharide moiety, B being the base moiety, the phosphoric acid moiety being attached at the 3' and/or the 5' position of the sugar moiety when said nucleotide is a deoxyribonucleotide and at the 2', 3' and/or 5' position when said nucleotide is a ribonucleotide, said base being a purine or a pyrimidine, said base being attached from the N1 position or the N9 position to the 1' position of the sugar moiety when said base is a pyrimidine or a purine, respectively, and wherein said Sig is a chemical moiety covalently attached to the base B of said nucleotide, said Sig when attached to said base B being capable of signalling itself or makes itself self-detecting or its presence known.**

[7] **Original claim 28 also recites a fluorescing component:**

> **28. A nucleotide in accordance with Claim 1 wherein said Sig chemical moiety comprises a component selected from the group consisting of an electron dense component, a magnetic component, an enzyme, a hormone component, a**

(continued...)

(b) ***Original claim 43***[8]

43. A nucleotide in accordance with Claim 42 wherein said fluorescing component is ***fluorescein***.

(c) ***Original claim 88***

88. A nucleotide in accordance with Claim 42 wherein said fluorescing component is ***rhodamine***.

(d) ***Original claim 89***

89. A nucleotide in accordance with Claim 42 wherein said fluorescing component is ***dansyl***.

(e) ***Original claim 130***

130. A ribonucleotide in accordance with Claim 101[9] wherein said Sig chemical moiety includes or comprises a ***fluorescing component*** attached thereto.

(f) ***Original claim 131***

131. A ribonucleotide in accordance with Claim 130 wherein said ***fluorescing component*** is ***fluorescein***.

(g) ***Original claim 132***

132. A ribonucleotide in accordance with Claim 130 wherein said ***fluorescing component*** is ***rhodamine***.

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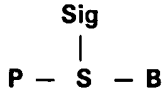
radioactive component, a metal-containing component, a fluorescing component and an antigen or antibody component. [underlining added]

[8] The exact language in original claim 42 is also recited in original claim 87. The latter claim is thus, a duplicate of claim 42, and has not been included here.

[9] Claim 101 recites:

101. A ribonucleotide having the general formula,



wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety, the phosphoric acid moiety being attached at the 2', 3' and/or 5' position of the sugar moiety, said base B being attached from the N1 position or the N9 position to the 1' position of the sugar moeity when said base is a pyrimidine or a purine, respectively, and wherein said Sig is a chemical moiety covalently attached to the sugar S, said Sig, when attached to said sugar S, being capable of signalling itself or making itself self-detecting or its presence known.

(h) Original claim 133

133. A ribonucleotide in accordance with Claim 130 wherein said ***fluorescing component*** is ***dansyl***.

The text of the eight originally filed claims above reasonably conveys to me that fluorescing components, including fluorescein, rhodamine and dansyl, are labels that are attached to nucleic acids.

THE '069 SPECIFICATION DISCLOSES DIFFERENT FLUORESCENT LABELS AS RECITED IN CLAIM 1796.

12. As a person skilled in the art, it is my opinion that the '069 specification discloses the different fluorescent labels which are recited in claim 1796.

A (i) Different fluorescent labels clearly disclosed in Example 9.

As set forth in Paragraph 11A(i) above, Example 9[10] discloses that several labeled clones are obtained for each chromosome from a human gene library of some "100 to 200 clones." Here, the '069 specification states:

> By allowing ***one set of labeled clones*** to hybridize to the chromosomes and then adding a ***fluorescent stain to the label***, the set of clones and their locations can be visualized and will ***flouresce***[sic] ***with a particular color***. A ***second set of labeled clones*** could then be used and reacted with a ***second fluorescent dye***. The same process can be repeated a number of times. Thus one can, if desired, have ***several sets of fluorescent labels attached to the cellular DNA*** at different but specific locations on each of the chromosomes.[11] [emphasis added]

The above-quoted passage clearly conveys to me as a person skilled in the art that several sets of fluorescent labels are attached to DNA and that these labels will flouresce with particular colors.

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[10] **'069 specification, page 47, 6th line from the bottom of the page.**
[11] **'069 specification, page 47, lines 5-14.**

A (ii) Different fluorescent labels are also originally claimed in the '069 specification.

As explained in Paragraph 11A(ii) above, three different fluorescent compounds -- fluorescein, rhodamine and dansyl -- are originally claimed in the '069 application. As explained in Paragraph 14B below, these three fluorescent compounds give off different colors that allow them to be distinguished from each other.

FLUORESCENT LABELS ARE SYNONYMOUS WITH FLUOROPHORES.

13. As also explained above,[12] the '069 specification reasonably conveys to me as a person skilled in the art that fluorescent labels and different fluorescent labels are attached to nucleic acids. Fluorescent labels are synonymous with fluorophores. In this instance, Campbell[13] provides the following definition for fluorophore:

> **Fluorophore:** A ***fluorescent*** substance. [emphasis added]

A more elaborate definition is provided by The Oxford English Dictionary:[14]

> **fluorophor, fluorophore** Also fluorphor. a. An atomic group the presence of which in a molecule causes it to be fluorescent. b. A ***fluorescent*** substance. [underlined emphasis added]

Stenesh[15] provides this definition:

> **fluorophore** A potentially ***fluorescent*** group of atoms in a molecule. [emphasis added]

Thus, the potentially fluorescent group of atoms in a fluorophore molecule are also present in fluorescent molecules and fluorescent labels.

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[12] See Paragraphs 11A(i), 11A(ii), 12A(i) and 12A(ii).
[13] Campbell, A. J., Chemiluminescence: Principles and Applications in Biology and Medicine, Ellis Horwood Ltd., Chichester, England, 1988, Appendix I, page 562; copy attached as Exhibit C.
[14] The Oxford English Dictionary, Second Edition, Clarendon Press, Oxford, England, 1989, page 1105; copy attched as Exhibit D.
[15] Stenesh, cited *supra*., page 180, copy attached as Exhibit E.

DIFFERENT FLUORESCENT LABELS EXHIBIT DIFFERENT SPECTRAL CHARACTERISTICS.

14. A. The different fluorescent labels recited in claim 1796 are fluorophores, and being of different colors, they too, exhibit different spectral characteristics. The particular color referenced in the '069 specification[16] refers to the particular spectral characteristics of that fluorescent compound. It is known in the art that the color of a fluorescent compound is dependent upon the emission wavelength of an *excited* fluorescent compound. A good description of these features is provided by Barrett:[17]

> The fluorescent technique makes use of special dyes referred to as fluors or fluorochromes. ***Fluors are chemical substances that are capable of absorbing a short wavelength of light and instantaneously emitting a longer wavelength light***. The dyes used for fluorescent antibody absorb in the ultraviolet and short blue range (200 to 400 nm) and emit a visible light. ***The exact absorption spectrum of the fluor and that of its emitted light are characteristic for each fluor***. The ***color of the emitted light is not a characteristic of the excitation light***.
>
> [emphasis added]

It is also well known in the art that identification using the color properties of fluorescent dyes is based on differences in fluorescence spectral characteristics between the dyes.[18,19,20]

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[16] '069 specification, Example 9, Section I (Karyotyping), page 47, line 9.

[17] Barrett, James T., Textbook of Immunology: An Introduction To Immunochemistry And Immunobiology, 4th Edition, The C. V. Mosby Company, St. Louis, MO, 1983, page 282; copy of pages 282-283 attached as Exhibit F.

[18] See four web pages from Olympus FluoView Resource Center (http://www.olympusfluoview.com/applications/flimintro.html) ("Conventional fluorescence microscopy makes use of the color properties of fluorescent dyes, that is, identification is based on differences in fluorescence spectral characteristics between dyes."); copy attached as Exhibit G.

[19] See also web page from "Spectral and Lifetime Imaging" (http://www.loci.wisc.edu/optical/specdec.html) ("The use of multiple fluorescent labels has long been commonplace in the study of fixed specimens, and is now becoming established for in vivo studies. Not so long ago only three fluorophores were in widespread use (fluorescein, rhodamine and DAPI); now there is a plethora of fluorophores available, each with its own unique spectral characteristics [underline in the original]."); copy attached as Exhibit H.

[20] See also two web pages from "New Methods for Karyotyping," (http://www.biology.arizona.edu/human_bio/current/new_karyotyping/new_karyotyping.html) ("The

(continued...)

B. As explained in Paragraph 11A(ii), three specific fluorescent compounds or dyes -- fluorescein, rhodamine and dansyl -- are recited in originally filed claims in the '069 specification. Barrett[21] describes all three:

> The ***fluorochromes*** usually chosen are ***fluorescein***, a ***rhodamine*** such as lissamine rhodamine B, and 1-dimethylaminonaphthalene-5 sulfonic acid (***DANSYL***). (Fig. 14-4). One or another of these is chosen because, although each fluoresces with high efficiency, a proper color is needed to avoid confusion with the blue-gray autofluorescence of tissues. ***Fluorescin and DANSYL give off a green or yellow-green light, and rhodamine gives off an orange-red hue***. . .
>
> [emphasis added]

As a person skilled in the art, I respectfully point out -- and this is explained by Barrett in the above-quoted passage -- that the spectral characteristics of fluorescein, rhodamine and dansyl allow one to distinguish these fluorescent compounds from each other on the basis of the particular colors given off by each.

15. In summary, the '069 specification conveys to me that fluorescent labels are attached to nucleic acids, and further, that different fluorescent labels are likewise attached to nucleic acids. Moreover, the '069 specification conveys to me that such different fluorescent labels attached to nucleic acids possess different spectral characteristics.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful

---

new karyotyping methods introduced by Schrock *et al* use fluorescent dyes that bind to specific regions of chromosomes. By using a series of specific probes each with varying amounts of the dyes, different pairs of chromosomes have unique spectral characteristics [underline added]."); copy attached as Exhibit I.

[21] Exhibit J, Page 282, left column, last paragraph, through first paragraph, right column.

false statements may jeopardize the validity of the application or any patent issued thereon.

9/14/04
Date

Dr. James J. Donegan

* * * * * * *

FinalDecl.9.14.04(5PM)

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Education

Ph.D, SUNY at Stony Brook, Stony Brook, NY 1981
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Experience

Senior Research Investigator at Enzo Life Sciences, Inc., Farmingdale, NY 1993-present
- Design and synthesis of recombinant DNA constructs for gene therapy
- Enhancement of introduction and expression of DNA in mammalian cells
- Novel methods of nucleic acid amplification
- Novel methods of gene construction

Staff Scientist at Genprobe, Inc., San Diego, CA 1992-1993
- Non-radioactive DNA probe assay development for proprietary amplification system
- Team leader with four research associates

Senior Scientist at Syngene, Inc., San Diego, CA 1991-1992
- Non-radioactive DNA probe assay system

Associate Investigator at Enzo Diagnostics, Inc., Farmingdale, NY 1984-1991
- Development of diagnostic assays
- Isolation and characterization of species specific DNA probes
- Modification of cloned probes for increased efficiency of production
- Novel vector construction
- RFLP analysis as a means of bacterial speciation
- Development of methodologies for signal amplification
- Assistance to attorneys in prosecution of patent applications
- Supervision of five technicians

Post-doctoral research associate with M. Inouye at SUNY at Stony Brook 1983-1984
- Development of methods to isolate species specific probes

Post-doctoral research associate with M. Freundlich at SUNY at Stony Brook 1982-1983
- Maxam-Gilbert sequencing of the ilv B gene

Post-doctoral associate with H. Ozer at Hunter College, New York, NY 1979-1982
- Characterization and chromosomal mapping of a mammalian temperature sensitive DNA synthesis mutant

Graduate student with R. Sternglanz at SUNY at Stony Brook 1974-1979
Bacteriophage T7 DNA replication
Assymetric characteristics of replication forks
Role of various host mutations on T7 DNA replication

Industrial Publications:

Stable Human Immunodeficiency Virus Type 1 (HIV-I) Resistance in Transformed CD4+ Monocytic Cells Treated with Multitargeting HIV-1 Aritisense Sequences Incorporated into U1 snRNA. Liu, D., Donegan, J.J., Nuovo, G., Mitra, D. and Laurence, J. (1997) J. Virology 71, 4079-4085.

High level and Stable HIV-1 resistance in CD4+ cells by Multi-targeting Antisense RNA incorporated into U1 snRNA. Liu, D., Donegan, J.J., Kelker, N., Nuovo, G; and Laurence, J. (1996) XI International Conference on AIDS, Vancouver, British Columbia, Canada.

Detection of Human Papillomaviruses Type 16 and 18 from clinical samples using Transcription Mediated Amplification. Gordon, P., Donegan, J.J., Sherrill, S., Deveze-Doyle, S., Doi, H., Hammond, P., Carter, N., Brentano, S., and McDonough, S. (1995) San Diego Conference on Nucleic Acids, San Diego, CA.

Non-radioactive, colorimetric microtitre plate assay for detecting of amplified Human immunodeficiency virus DNA. Rapier, J.L., Villamarzo, Y., Schochetman, G., Ou, C.Y., Brakel, C.L., Donegan, J.J., Maltzman, W., Lee, S., Kirtikar, D. and Gatica, D. (1993) Clinical Chemistry 39, 244-247.

The use of non-radioactive probes for rapid diagnosis of sexually transmitted bacterial infections. Goltz, S., Donegan, J.J., Yang, H.L., Pollice, M., Todd, J.A., Molina, M.M., Victor, J. and Kelker, N. (1990) in Gene Probes for Bacteria, A.J.L. Macario and E.C, deMacario eds. Academic Press, Inc.

Isolation of a species specific probe for Neisseria gonorrhoea using a novel technique particularly suitable for use with closely related species displaying high levels of DNA homology. Donegan, J.J., Lo, A,., Manwell, A., Picken, R., and Yang. H. L. (1989) Molecular and Cellular Probes 3, 13-26.

DNA Probes for mycobacteria I. Isolation of DNA probes for the identification of Mycobacterium tuberculosis complex and for mycobacteria other than tuberculosis (MOTT). (1988) Picken, R.N., Plotch, S.J., Wang, Z., Donegan, J.J., and Yang, H.L. Molecular and Cellular Probes 2, 111-124.

An automatable colorimetric DNA hybridization test for mycobacterium tuberculosis confirmation (1987) Brakel, C.L., Donegan, J.J., Linn, C.I.P., Molina, M., and Pollice, M.A. Abstracts of the Annual Meeting of the American Society for Microbiology 88., 144.

Detection of HPV by in situ hybridization with biotinylated DNA probes (1987) Todd, J., Jou, L., Donegan, J.J., Yang, H.L. 76th Annual Meeting of the United States and Canadian Academy of Pathology, Chicago, Ill., USA.

Academic Publications

The ilvB locus of E. coli K12 is an operon encoding both subunits of acetohydroxyacid synthase, Friden, P., Donegan, J.J., Mullen, J., Tsui, P., Freundlich, M., Eoyang, L., Weber, R. and Silverman, P.M. Nucleic Acids Research 13 pp. 3979-93, 1985.

Characterization of 8 ts mutant Balb-3T3 cells and correction of the defect by in vitro addition of extracts from wild type cells. Zang, G.C., Donegan, J.J., Ozer, H.L., and Hand. R. Molecular and Cellular Biology 4. 1815-22, 1984.

Evidence that both growing DNA chains at a replication fork are synthesized discontinuously, Rolf Sternglanz, Helen F. Wang, and James J. Donegan, Biochemistry 15., pp. 1838-43, 1976.

Evidence that both growing DNA chains at a replication fork are synthesized discontinuously. Rolf Sternglanz, Helen F. Wang and James J. Donegan, pp. 309-21 in DNA Synthesis and its Regulation, M. Goulian and P. Hanawalt, eds., W.A. Benjamin, Inc., 1976.

Issued U.S. Patents

Rabbani, et al., U.S. Patent No. 6,764,821, Detecting the Presences of Specific Target Nucleic Acid Sequences Through Stem-Loop Formation

Rabbani, et al., U.S. Patent No. 6,743,605, Linear Amplification of Specific Nucleic Acid Sequences

Engelhardt et al., U.S. Patent Appl. Serial No. 08/486,069 (Filed June 7, 1995)
Claims 1795-1796 For Submission
Exhibit B To Supplmental Declaration Of Dr. James J. Donegan

Claim 1795 (NEW). A process for determining the sequence of a nucleic acid of interest comprising:

providing or generating detectable non-radioactively labeled nucleic acid fragments comprising: (a) a sequence complementary to said nucleic acid of interest or a portion thereof, and (b) fluorescent labels covalently attached, directly or through a linkage group, to said fragments;

subjecting said labeled fragments to a sequencing gel to separate or resolve said labeled fragments;

detecting non-radioactively said separated or resolved fragments by means of said attached fluorescent labels; and

determining the sequence of said nucleic acid of interest from said detected fragments.

Claim 1796 (NEW). A process for determining the sequence of a nucleic acid of interest comprising:

providing or generating detectable non-radioactively labeled nucleic acid fragments comprising: (a) a sequence complementary to said nucleic acid of interest or a portion thereof, and (b) different fluorescent labels covalently attached, directly or through a linkage group, to said fragments;

subjecting said labeled fragments to a sequencing gel to separate or resolve said labeled fragments;

detecting non-radioactively said separated or resolved fragments by means of said attached different fluorescent labels; and

determining the sequence of said nucleic acid of interest from said detected fragments.

* * * * * * *

Campbell

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A. K. Campl

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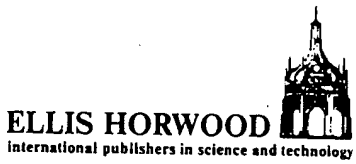

Appendix I

A glossary of some terms associated with luminescence

These are not necessarily intended as 'dictionary' definitions, rather a guide as to how I have used these terms in this book.

Anodoluminescence: Radioluminescence caused by positive 'rays' (α particles).
Apoprotein: protein without its prosthetic group e.g. apo-photoprotein.
Bioluminescence: Visible light emission from luminous organisms. All known examples are biological chemiluminescence. Also used to describe reactions extracted from luminous organisms e.g. in bioluminescent assays.
Candoluminescence: Luminescence in incandescent solids.
Cathodoluminescence: Radioluminescence caused by cathode 'rays' (β particles).
Charge-coupled device: a solid state device (pixels per square cm) which produces a charge when hit by a photon. The array is then reset by a computer which reconstructs the image.
Charge-transfer complex (CTC): Complex between two molecules of opposite charge (A^+B^- or A^+A^-). The resulting energy can give rise to luminescence.
Chemically initiated electron-exchange luminescence (CIEEL): Luminescence resulting from a process involving electron exchange between atoms or molecules, initiated by a chemical reaction.
Chemienergisation: Process where energisation of an atom or molecule arises from a chemical reaction.
Chemiexcitation: Process where excitation of an atom or molecule arises from a chemical reaction, i.e. the key step in a chemiluminescent reaction.
Chemiluminescence: The emission of light (strictly electromagnetic radiation in the UV, visible and IR) as a result of a chemical reaction. The enthalpy of reaction gives rise to an atom or molecule in a vibronically excited state, which then emits a photon on decaying to ground state.
Chemiluminescent reaction: The complete reaction of an atom or molecule to form products, producing light. One of the steps will involve *chemiexcitation*.

Einstein: The number of photons equivalent to 1 mole (i.e. 6.023×10^{23} photons).

Electrochemical luminescence (EL): Chemiluminescence resulting from electrolysis.

Electrochemiluminescence (ECL): Chemiluminescence, in solution, resulting from high energy electron transfer reactions.

Electrogenerated chemiluminescence (EGCL): Electrochemiluminescence.

Electroluminescence: Luminescence resulting from an electric current or discharge.

Electron exchange: Exchange of electrons between atoms or molecules, usually close enough for molecular orbital overlap. If one of the pair is an electronically excited state, it may thereby transfer its energy to an electron in the other atom or molecule.

Electronically excited state: An atom or molecule where an electron is raised into an orbital of higher energy. It is thus unstable. One mechanism for loss back to ground state is photon emission (luminescence).

Electron transfer: The transfer of an electron from one molecule to another. Its occurrence during radical annihilation or charge transfer complexes provides a mechanism for both generating an electronically excited state or for transferring energy from one atom or molecule to another.

Energy transfer: The transfer of energy from one atom or molecule to another. In luminescence, it involves transfer from an excited state donor to an acceptor which itself becomes excited, and then emits. It is usually used only for non-radiative processes.

Excimer: A charge-transfer complex between two molecules of the same chemical structure (A^+A^-). Neutral complex (AA) is also possible.

Exciplex: A charge-transfer complex between two molecules of different chemical structure (A^+B^-). Neutral complex (AB) is also possible.

Excited state: Electronically excited state as used in this book.

Extracellular: Outside a cell.

Filter: In a luminous organism this refers to a layer of pigmented cells or an acellular pigment, through which the light passes. The colour of the light emanating from the organism is thus different from that from the photocytes, because of absorption by the pigment.

Fluorescence: A form of photoluminescence where the electronically excited state is generated by absorption of light (UV or visible). The emission is from the same spin state as the ground state (usually singlet to singlet). The life time of a fluorescent atom or molecule is very fast, usually 1–30 ns, though some longer-lived species are known.

Fluorophore: A fluorescent substance.

Förster mechanism (or energy transfer): Resonance energy transfer.

Franck–Condon principle: The principle by which absorption of energy by a chemical bond, e.g. from a photon, is so fast that it occurs without a change in separation between the nuclei, i.e. in a potential energy well diagram the path to the excited state is vertical.

Galvanoluminescence: An old term, a form of electroluminescence, when solutions are electrolysed.

Hydroid: The fixed state of a hydrozoan jelly fish (phylum Cnidaria). N.B. Some hydroids have no free-floating stage.

Image intensifier: A
its detector.
does not inv
Incandescence: Em
The energy
between rat
emission ob
Indicator-dependen
reaction of
pound, the
Indirect chemilumir
Intermolecular: Bet
Intersystem crossing
state to ano
Intracellular: Inside
Intramolecular: Wit
Iridocyte: A reflecti
Iridophore: Reflecti
Lens: In the eye, a tr
or luminou
organism.
Light intensity (I): T
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the brightne
observed.
Light organ: Multice
Living light: Biolum
Luciferase: The prot
term is norm
luciferase, t
Luciferin: The chen
book, I have
sible for the
photoprotei
Luminescence: The
electronicall
tinguish phe
descence, i.e
Luminescent chromc
Luminescent reactio
Luminometer: Appa
phenomeno
miluminesce
Luminous organism:
Lumiphore: A substr
Lux gene: A gene wit
the marine b

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SECOND EDITION

Prepared by

J. A. SIMPSON *and* E. S. C. WEINER

VOLUME V

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forming, with bromine, chlorine, and iodine, the halogen group.

1813 SIR H. DAVY in *Phil. Trans.* CIII. 278 It appears reasonable to conclude that there exists in the fluoric compounds a peculiar substance..it may be denominated fluorine, a name suggested to me by M. Ampère. 1869 ROSCOE *Elem. Chem.* 13 All the elements, with the single exception of fluorine, combine with oxygen to form oxides.

2. *attrib.*, as **fluorine dating, method, test**, etc., a method of determining the relative age of organic remains by measuring the amount of fluorine that has been absorbed from surrounding ground-water.

1949 OAKLEY & MONTAGU in *Bull. Brit. Mus.* (*N.H.*), *Geol.* I. II. 29 The authors..independently reached similar conclusions with regard to the probable dating of the [Galley Hill] skeleton and..prepared a joint report on their findings. One author (K.P.O.) has prepared the introductory sections, the account of the geology, and of the fluorine dating. 1959 J. D. CLARK *Prehist. S. Afr.* iv. 83 Fluorine and uranium tests have shown that the skull fragments are of the same age as the faunal remains found with them. 1968 R. G. WEST *Pleistocene Geol. & Biol.* ix. 160 The fluorine method is useful for the relative dating of animal skeletal remains found in sand and gravel.

fluorite ('flu:əraɪt). *Min.* [f. FLUOR + -ITE.] = FLUOR-SPAR.

1868 DANA *Min.* p. xxx, Fluor was written fluorite last century by Napione. 1887 —— *Min. & Petrogr.* 228 Massive fluorite receives a high polish.

fluoro- (flu:ərəʊ). 1. Used as comb. form of FLUORINE (rarely of FLUORIDE), chiefly in the names of chemical compounds, as **fluoroa'cetamide**, $CH_2F{\cdot}CONH_2$, a stable, toxic, fluorinated derivative of acetamide with strong insecticidal properties; **'fluoroform** [ad. F. *fluoroforme* (Meslans 1890, in *Compt. Rend.* CX. 717): see CHLOROFORM], a gas (CHF_3) that is almost completely inert both chemically and physiologically and is the fluorine analogue of chloroform; **,fluorohydro'cortisone** = FLUDROCORTISONE; **'fluorotype** *Photogr.* [-TYPE], an old positive process in which paper sensitized with sodium fluoride was used.

1909 *Jrnl. Chem. Soc.* XCVI. II. 297 The author has determined the heats of formation of the following compounds... *Fluoroacetamide, 249·55 cal. 1958 *Nature* 28 June 1810/2 Fluoroacetamide is as effective as the more dangerous sodium fluoracetate as a systemic insecticide. 1964 *New Statesman* 20 Mar. 438/3 Last month the government banned the sale of fluoroacetamide as an insect control. 1890 *Jrnl. Chem. Soc.* LVIII. II. 724 This gas is *fluoroform, CHF_3. 1950 N. V. SIDGWICK *Chem. Elements* II. vii. 1130 A guinea-pig put into a 50 per cent. air-fluoroform mixture..did not know that the gas was there. 1954 FRIED & SABO in *Jrnl. Amer. Chem. Soc.* LXXVI. 1455/2 9α-*Fluorohydrocortisone acetate..was obtained in about 50% yield. *Ibid.* 1456/1 Deacetylation of I [*sc.* 9α-fluorohydrocortisone acetate] with sodium methylate afforded 9α-fluorohydrocortisone. 1970 W. MODELL *Drugs of Choice 1970-71* xxxii. 511 All patients with Addison's disease now can be satisfactorily managed with oral therapy using 10 mg. of hydrocortisone 2 to 3 times a day, plus 0·1 mg. of 9-alpha-fluorohydrocortisone (fludrocortisone) a day or every other day. 1844 R. HUNT *Res. Light* 106 It has been found that the fluate of soda has the property of quickening the sensibility of bromidated papers to a very remarkable extent; and from this quality a new process, which I would distinguish by the name of the *Fluorotype, results. 1955 H. & A. GERNSHEIM *Hist. Photogr.* III. xi. 124 Fluorotype derives its name from the fluoride of sodium used in preparing the paper... The exposure was only half a minute and the picture was developed with protosulphate of iron.

2. Used as combining form of FLUORESCENCE, as in FLUOROMETER, etc.

fluorocarbon (,flu:ərəʊ'kɑ:bən). *Chem.* [f. FLUORO- + CARBON, after *hydrocarbon*.] Any of a large class of synthetic, chemically stable compounds of carbon and fluorine analogous to the hydrocarbons (see also quot. 1962).

1937 *Jrnl. Amer. Chem. Soc.* LIX. 1407/1 From a reaction mixture of carbon and fluorine..fluorocarbons have been isolated. 1950 J. H. SIMONS *Fluorine Chem.* I. xii. 402 Substances made from or protected by fluoro-carbons are free from decay and insect damage. 1951 *Sci. News Letter* 15 Sept. 165 The relatively new chemicals known as fluorocarbons when used as a cooling spray greatly increase the efficiency of electrical transformers. 1959 *Times* 27 Apr. (Rubber Industry Suppl.) p. vi/7 The newer fluoro-carbon polymers have valuable properties in heat and fluid resistance. 1962 A. J. RUDGE *Manuf. Fluorine* vii. 57 In the technical literature there has developed a tendency to use the term 'fluorocarbon' to include compounds containing elements additional to carbon and fluorine, e.g. CF_2Cl_2. This practice is confusing and is to be deprecated. 1964 R. E. BANKS *Fluorocarbons* v. 136 Like their aliphatic relatives, aromatic fluorocarbons are colourless. 1966 *New Scientist* 24 Nov. 456/2 Fluorocarbons such as polytetrafluoroethylene (PTFE) and related polymers.

fluoroid ('flu:ərɔɪd). *Crystallogr.* [f. FLUOR + -OID.] A solid bounded by twenty-four triangular planes; occurring frequently in fluor-spar.

fluorometer (flu:ə'rɒmɪtə(r)). [f. FLUORO- + -METER.] 1. A device used to aid in the fluoroscopic location of one object within another.

1897 *N.Y. Tribune* 9 Feb. 3/4 In a series of scientific experiments with the new fluorometer, invented by John Dennis of this city [*sc.* Rochester], Professor A. L. Arey demonstrated definitely the angles and direction of the Roentgen rays with regard to their source. 1898 *Sci. Amer.* 12 Feb. 101/1 It is the province of the 'fluorometer' to enable observers to form an exact and certain diagnosis of the presence of bullets, needles, calculi or any other substance which is comparatively more dense in its fluoroscopic shadow than the subject in which it is contained. 1899 D. WALSH *Röntgen Rays in Med. Work* (ed. 2) 97 In America the 'Dennis Fluorometer' is in vogue.

2. Also **fluorimeter**. Any apparatus or instrument for measuring the intensity of fluorescence or the duration of its afterglow. Hence **fluoro-, fluori'metric** *adjs.*, of, pertaining to, or employing the fluorometer or fluorometry; **fluoro-, fluori'metrically** *advs.*; **fluo'ro-, fluo'rimetry**, the use of the fluorometer.

1913 *Chem. Abstr.* VII. 2905 The instrument described, termed a fluorometer. 1918 *Ibid.* XII. 450 (*heading*) New fluorometric apparatus for the determination of X-rays. 1920 *Ibid.* XIV. 2584 (*heading*) A fluorimeter. 1920 *Jrnl. Amer. Chem. Soc.* XLII. 1351 The natural term to designate the new method could be either 'fluorometry' or 'fluoremetry' [*sic*]. 1942 *Electronic Engin.* XV. 127 A supersonic cell fluorometer..for the measurement of the rise and decay of luminescence in phosphors. 1953 BOWEN & WOKES *Fluorescence of Solutions* viii. 58 The term 'fluorimeter'..is now generally accepted in Great Britain. Alternative terms such as 'fluorometer' and 'fluorophotometer' are in use in America. 1961 *Lancet* 7 Oct. 793/1 The urine was..estimated fluorimetrically. 1962 T. O. SIPPEL in A. Pirie *Lens Metabolism in Rel. Cataract* 368 The oxidized and reduced forms of diphospho- and triphosphopyridine nucleotide were measured fluorometrically. *Ibid.*, Readings were made on a photomultiplier fluorometer.

fluorophor, fluorophore ('flu:ərəʊfɔə(r)). Also fluorphor. [a. G. *fluorophor* (R. E. Meyer 1897, in *Zeitschr. f. physiol. Chem.* XXIV. 508), f. FLUORO- + -PHORE.] a. An atomic group the presence of which in a molecule causes it to be fluorescent. b. A fluorescent substance.

1898 *Jrnl. Chem. Soc.* LXXIV. II. 105 In order..that the fluorescence may be developed, it is further necessary that the fluorphore be situated between two heavy atomic groups, usually benzene nuclei. 1939 RADLEY & GRANT *Fluorescence Anal. U.V. Light* (ed. 3) II. xiv. 290 Some fluorescent compounds of simple composition but containing no 'fluorophors' are known. 1962 S. UDENFRIEND *Fluorescence Assay* ii. 33 Nonfluorescent steroids are converted to fluorophores by dehydration in concentrated sulfuric acids. 1966 *McGraw-Hill Encycl. Sci. & Technol.* VII. 611/1 Other terms sometimes used synonymously with phosphor are luminophor..or fluorphor.

fluorophotometer (,flu:ərəʊfəʊ'tɒmɪtə(r)). Also ,fluopho'tometer, ,fluorpho'tometer. [f. FLUORO- + PHOTOMETER.] A fluorometer (sense 2) incorporating a photometer. Hence **,fluorophoto'metric** *a.*, of, pertaining to, or employing a fluorophotometer or fluorophotometry; **,fluoropho'tometry**, the use of the fluorophotometer.

1928 *Jrnl. Sci. Instrum.* V. 273 (*heading*) A simple ultra-violet fluorophotometer. 1946 *Nature* 28 Sept. 451/2 The result of the 'fluorimetry' or 'fluorphotometry' of samples will depend on their previous history as regards exposure to light. 1950 *Arch. Ind. Hyg. & Occup. Med.* II. 311 Fluorophotometric analysis for uranium. 1953 Fluoro-photometer [see FLUOROMETER 2]. 1969 G. W. EWING *Instrum. Meth. Chem. Analysis* (ed. 3) iv. 107 Instruments for the measurement of fluorescence are known as fluorimeters (sometimes fluorometers or fluophotometers).

fluoroscope ('flu:ərəʊskəʊp). [f. FLUORO- + -SCOPE.] An apparatus which incorporates a fluorescent screen and is used in conjunction with an X-ray machine to produce a visible image of a body placed between the screen and the source of the rays. Hence **fluoro'scopic** *a.*, formed or done by means of a fluoroscope or fluoroscopy; pertaining to the fluoroscope or fluoroscopy; **fluoro'scopically** *adv.*; **fluo'roscopy**, the use of the fluoroscope; an examination by means of a fluoroscope.

1896 *Lancaster* (Pa.) *New Era* 2 Apr. 2 He [*sc.* Edison] calls his instrument the Fluoroscope. 1896 *Boston Med. & Surg. Jrnl.* 1 Oct. 336/1 A fluoroscopic examination of the heart. *Ibid.* 335/1 The constant motion of the heart and diaphragm interfere with the use of radiography but renders fluoroscopy all the more valuable. 1897 *Chem. News* 24 Sept. 158/1 (*heading*) Photography of the fluoroscopic image. 1908 *Practitioner* Sept. 437 Fluoroscopic examination of the thorax was also negative. 1940 G. L. CLARK *Applied X-Rays* (ed. 3) ix. 166 A typical unit for continuous fluoroscopic inspection of..food products on the conveyor belt. 1959 *Medicamundi* V. 4 (*heading*) Fluoroscopically controlled cholangiography with the image intensifier. 1961 A. TAYLOR *X-Ray Metallogr.* iii. 32 The smaller castings made from light alloys are usually examined for major defects by fluoroscopic inspection. 1970 *Nature* 18 July 296/1 In many hospitals the records will be sufficiently accurate to determine the number of fluoroscopies per patient treated, as well as radiation dose per fluoroscopy.

fluorosis (flu:ə'rəʊsɪs). *Path.* [ad. F. *fluorose* (H. Christiani 1927, in *Compt. Rend. Sixième Congrès Chim. Ind.* 164/1), f. FLUOR- + -OSIS.] Poisoning by fluorine or a fluorine compound; any condition caused by such poisoning.

1927 *Chem. Abstr.* XXI. 3404 An investigation which resulted in characterizing a new disease, fluorosis. 1936 *Nature* 16 May 828/2 Fluorine from chemical works or resulting from volcanic activity can get into soil and pasture and cause fluorosis in cattle. 1958 *Spectator* 6 June 737/2 Crippling fluorosis in *natural* fluoride areas at or near the so-called safe concentration has been admitted by some of the top American proponents them-selves. 1971 *Daily Tel.* (Colour Suppl.) 28 May 21/4 Cattle nearby have in the past suffered from fluorosis, a condition not unlike rheumatoid arthritis in which the joints seize up.

†**'fluorous**, *a. Obs.* [f. FLUOR + -OUS.] Only in *fluorous acid*: (see quot. 1828: no such acid exists).

1790 KERR tr. *Lavoisier's Elem. Chem.* 185 (Table), Fluorous acid. 1828 WEBSTER s.v. (citing LAVOISIER), The fluorous acid is the acid of fluor in its first degree of oxygenation.

fluorphor, var. FLUOROPHOR.

fluor-spar ('flu:əspɑ:(r)). *Min.* [f. FLUOR + SPAR.] Native fluoride of calcium ($Ca F_2$); found abundantly in Derbyshire (where one variety is known as *Blue John*), and hence often called *Derbyshire spar*.

1794 KIRWAN *Min.* I. 127, 3d Family, Foliated or sparry, Fluor spar. 1812 SIR H. DAVY *Chem. Philos.* 465 A substance found abundantly in nature called *fluor spar*, it is usually either blue, green, yellow, or white, transparent, and crystallized in cubes. 1880 ANSTED *Minerals* 18 The crystal of fluor-spar has the striæ parallel all round the four sides.

†**'fluoruret**. *Chem. Obs.* [f. FLUOR + -URET, q.v.] = FLUORIDE.

1854 J. SCOFFERN in *Orr's Circ. Sc.* Chem. 397 Fluorides or fluorurets.

†**flur**, *sb.*[1] *Obs. rare. Bird-catching.* 'A moveable perch to which a bird is tied and which the bird-catcher can raise by means of a long string.' (Pennant). Also *attrib.*, as *flur-bird*.

1766 PENNANT *Zool.* (1768) II. 331 He hath, besides, what are called flur-birds, which are placed within the nets, are raised upon the flur and gently let down at the time the wild bird approaches them. 1797 P. WAKEFIELD *Mental Improv.* (1801) I. 57 The flur-birds are braced by a silken string.

flur (flɜ:(r)), *sb.*[2] *Sc.* [? f. FLURR *v.*] Flue, fluff.

1845 *New Stat. Acc. Scot.* VI. 146 The dust and small flur separated from the cotton.

flur, obs. var. of FLOOR, FLURR.

flurdom, variant form of FLIRDOM.

fluren, obs. form of FLOUREN.

flures, flureis, fluris(che, obs. forms of FLOURISH.

flurn (flɜ:n), *v. Obs.* exc. *dial.* [? alteration of *flurre*, FLEER, after *spurn* or *scorn*.] *intr.* To sneer (*at*).

1656 R. FLETCHER *Ex Otio Negotium* To Rdr., And for those abortive births slipp'd from my brain..give me leave to flurn at them, as the poor excrescencies of Nature. 1866 BROGDEN *Prov. Words Lincolnsh.* 72 *Flurn*, to show contempt by looks, to scorn.

flurr, *sb. rare.* [f. next vb.] Flutter, whirr.

1651 H. MORE *Enthus. Triumph.* (1656) 208 After the flur and farre flight of every partridge he let out of his basket.

flurr (flɜ:(r)), *v.* [? onomatopœic.]

1. *trans.* To scatter, throw about; also with *up*.

1627-77 FELTHAM *Resolves* II. xxix. 218 Choler is as dust flur'd up into the eyes of Reason. 1813 HOGG *Queen's Wake* 39 The stately ship..flurred on high the slender spray.

2. *intr.* To fly up; to fly with whirring or fluttering wings.

1681 GLANVILL *Sadducismus* II. (ed. 2) 169 A Bird, that would flurr near to her face. 1824 *New Monthly Mag.* X. 322, I saw one [cuckoo]..flurr awkwardly away across the meadow. 1825 HOGG *Queen Hynde* 329 On the spray, that flurr'd and gleam'd A thousand little rainbows beam'd.

flurr(e, obs. f. FLEER.

†**flurred**, *a. Her. Obs.*[-1] [anglicized form of Fr. *fleuré, -ée*: see FLEURY.] Having flowerlike ornaments.

1655 M. CARTER *Hon. Rediv.* (1660) 86 A Coronet of gold flurred, the points and flowers of equall height.

flurri(e, obs. forms of FLEURY.

flurrifi'cation. *nonce-wd.* [f. FLURRY + -FICATION.] The state of being flurried.

1822 MRS. NATHAN *Langreath* II. 327 To be put into such a flurrification.

flurrish, obs. form of FLOURISH.

flurry ('flʌrɪ), *sb.* [? onomatopœic, suggested by *flaw, hurry* etc.; cf. also FLURR *v.*]

1. a. A sudden agitation of the air, a gust or squall.

1698 FRYER *Acc. E. India & P.* 128 *marg.*, Flurries from the Hills carry Men and Oxen down the Precipice. 1726-7

DICTIONARY OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Second Edition

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Professor of Chemistry
Western Michigan University

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excited and caused to fluoresce; subsequently the fluorescence is decreased when the antibody is allowed to combine with the hapten and an energy transfer takes place from the excited antibody to the hapten. The method can likewise be used to study binding reactions with other proteins.

fluorescence recovery after photobleaching FLUORESCENCE MICROPHOTOLYSIS.

fluorescent antibody An antibody that is covalently linked to a fluorescent dye, such as fluorescein or rhodamine, and that has retained its immunochemical activity.

fluorescent antibody technique A technique for locating either antigens or antibodies in a microscopic preparation of cells or tissues by treating the preparation with the corresponding fluorescent antibodies or fluorescent antigens. *See also* direct fluorescent antibody technique; indirect fluorescent antibody technique; anticomplement fluorescent antibody technique.

fluorescent antigen An antigen that is covalently linked to a fluorescent dye, such as fluorescein or rhodamine, and that has retained its immunochemical activity.

fluorescent screen A plate coated with a material, such as calcium tungstate or zinc sulfide, which fluoresces upon irradiation.

fluoridation The addition of fluoride to water supplies in an attempt to decrease dental caries; the final fluoride concentration is usually 1 mg/L.

fluorimeter Variant spelling of fluorometer.

fluorimetry Variant spelling of fluorometry.

fluorine An element that is essential to humans and animals. Symbol, F; atomic number, 9; atomic weight, 18.9984; oxidation state, −1; most abundant isotope, ^{19}F.

fluorochrome A substance that, when irradiated with light of a certain wavelength, emits light of a longer wavelength; a fluorescent compound, particularly one used to stain biological specimens.

1-fluoro-2,4-dinitrobenzene *See* Sanger reaction.

fluorography *See* solid scintillation fluorography.

fluoroimmunoassay An immunoassay employing antigens labeled with a fluorochrome. *Abbr* FIA.

fluorometer An instrument for the measurement of fluorescence that contains both a light source for supplying the excitation energy and a light detector for measuring the emission energy; filter fluorometers and spectrofluorometers are the two basic types.

fluorometry The measurement of fluorescence that may include a determination of one or more of the following: (a) the concentration of a fluorescent compound; (b) the relative efficiencies of various exciting wavelengths to cause fluorescence; (c) the relative intensities of various wavelengths in the emitted fluorescent light; and (d) the probability that an absorbed photon will generate an emitted photon in fluorescence.

fluorophenylalanine An amino acid analogue of phenylalanine that can be incorporated into protein during protein synthesis.

fluorophore A potentially fluorescent group of atoms in a molecule.

fluorosis A condition caused by excessive intake of fluorine, usually derived from drinking water, and characterized by the occurrence of mottled teeth.

5-fluorouracil A pyrimidine analogue that is used in cancer chemotherapy; an antitumor agent that inhibits the enzyme thymidylate synthetase. *Abbr* FU.

flush ends *See* restriction enzyme.

flu virus INFLUENZA VIRUS.

flux 1. The metabolic rate with respect to a particular substrate in a given tissue; equal to AV/K_m where A is the substrate concentration in the tissue, V is the maximum velocity, and K_m is the Michaelis constant. 2. The rate of flow of either matter or radiation; equal to the number of particles (or the mass) or the number of photons that pass through a unit area per unit time. *See also* glycolytic flux.

flux ratio method A technique that is useful for the interpretation of complexities in enzyme mechanisms. It resembles a product inhibition technique but, rather than examining effects on initial rates, it examines the fate of individual product molecules participating in inhibitory reactions. Thus, for the reaction $A + B \rightleftharpoons P + Q$, the ratio of two fluxes, one involving the conversion $P \rightarrow A$ and the other that of $P \rightarrow B$ can be determined and plotted as a function of the concentration of A or B, respectively. The resulting curves can be interpreted as supporting a random or an ordered mechanism.

F-mediated transduction SEXDUCTION.

fMet-tRNA *N*-Formylmethionyl tRNA.

FMN Flavin mononucleotide.

$FMNH_2$ Reduced flavin mononucleotide.

Fm protease A proteolytic enzyme, isolated from *Flavobacterium meningosepticum*, that cleaves peptide bonds in which the carbonyl group is donated by proline or methionine.

FNPA 4-Fluoro-3-nitrophenyl azide; a photoaffinity labeling compound that selectively binds to the active sites of protein molecules in antibodies and in acetylcholine binding sites on intact membranes.

foam The colloidal dispersion of a gas in a liquid.

foam cells Lipid-sw
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TEXTBOOK OF IMMUNOLOGY

AN INTRODUCTION TO IMMUNOCHEMISTRY AND IMMUNOBIOLOGY

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University of Missouri School of Medicine
Columbia, Missouri

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With **194** illustrations

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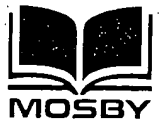

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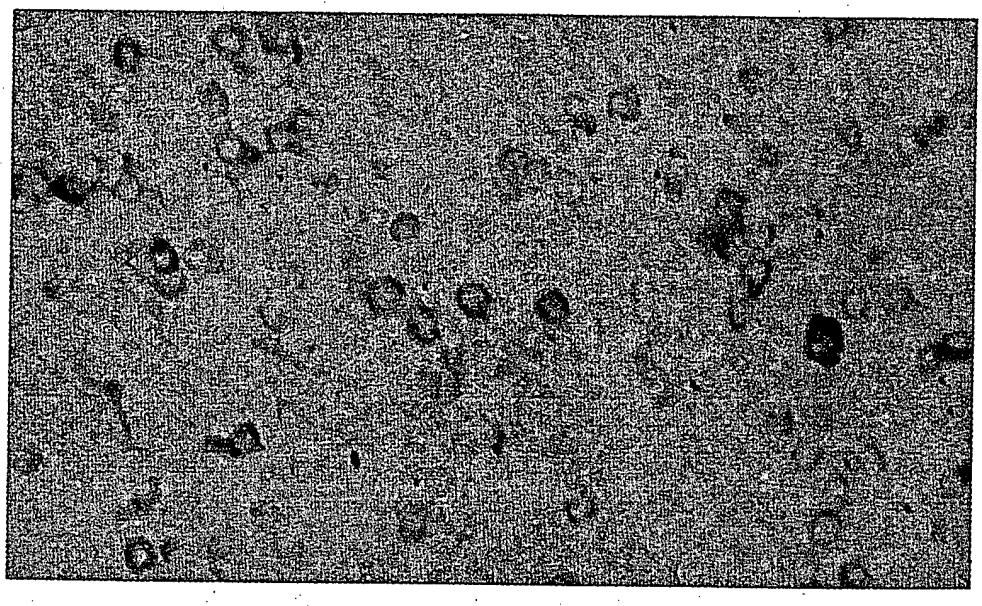


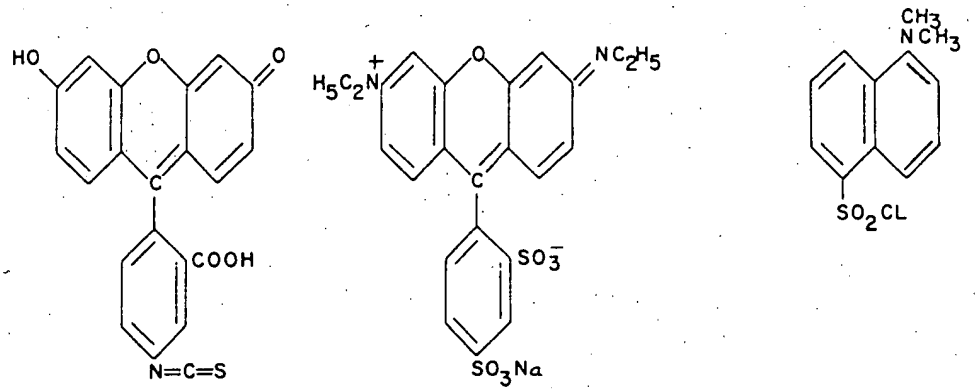
Fig. 14-4. The dark rings seen in this photograph represent the end product of an ELISA test in which a horseradish peroxidase–labeled antibody to human IgG was used to stain a preparation of lymphocytes. The enzyme substrate used was *o*-dianisidine, which forms an insoluble black product when oxidized. In the background the faint outline of cells that do not bear IgG on their surface is barely visible. (Courtesy Dr. E. Adelstein.)

azodyes, but little in the way of practical results came from their preparation and use. No true success was achieved until the early 1940s, when Coons developed the fluorescent antibody method.

The fluorescent technique makes use of special dyes referred to as fluors or fluorochromes. Fluors are chemical substances that are capable of absorbing a short wavelength of light and instantaneously emitting a longer wavelength light. The dyes used for fluorescent antibody absorb in the ultraviolet and short blue range (200 to 400 nm) and emit a visible light. The exact absorption spectrum of the fluor and that of its emitted light are characteristic for each fluor. The color of the emitted light is not a characteristic of the excitation light.

The fluorochromes usually chosen are fluorescein, a rhodamine such as lissamine rhodamine B, and 1-dimethylaminonaphthalene-5 sulfonic acid (DANSYL) (Fig. 14-5). One or another of these is chosen because, although each fluoresces with high efficiency, a proper color is needed to avoid confusion with the blue-gray autofluorescence of tissues. Fluorescein and DANSYL give off a green or yellow-green light, and rhodamine gives off an orange-red hue. All three are easily bonded to the free amino groups of the antibody molecule. Fluorescein ordinarily is purchased in the form of fluorescein isothiocyanate, which forms a thiocarbamido linkage with amino groups of protein. Rhodamines and DANSYL more often are prepared as sulfonyl chlorides, which form sulfonamido bonds with proteins. Since free amino groups of lysine are not especially critical to the activity of the antibody, the covalent bonding of these ligands does not destroy the antibody activity unless carried to excess.

The antibody preparation to be labeled should be a purified γ-globulin preparation, since most fluors will label albumin and even α- and β-glob-



Fluorescein isothiocyanate

Lissamine rhodamine B

1-Dimethylamino-naphthalene-5-sulfonyl chloride (DANSYL)

Fig. 14-5. Three fluorochromes often used in fluorescent labeling of antibodies. All three of these compounds couple to the amino groups of proteins. Under ultraviolet illumination fluorescein and DANSYL emit a green or yellow-green light and rhodamine a red-orange light.

ulins much better than γ-globulins. Unless these serum proteins are excluded, the fluorescent antibody preparation will suffer from the dual handicap of low fluorescence and nonspecific staining by the other labeled serum proteins. The precipitation, agglutination, or some other serologic titer of the γ-globulin fraction of the antiserum should be determined before and after labeling. The immunofluorescent behavior of an antiserum is not dependent exclusively on its precipitation or other titer, since monovalent antibodies also function as fluorescent antibodies; but it is important to know if a great loss of antibody activity occurred during the labeling procedure. Specific labeling directions will differ slightly for different fluors and generally are based on specific dye/protein ratios. Careful adherence to the directions is necessary to avoid losses of antibody activity and nonspecific staining caused by overlabeling. Unreacted fluor can be removed by gel filtration or dialysis. Dilutions of the labeled antibody then should be tested on known preparations to determine its activity and nonspecificity. Fluorescent antibody preparations with a high nonspecific background staining may be absorbed repeatedly with dried acetone powders of animal tissues to improve their quality. Background staining of tissue preparations with labeled albumin or simple dyes such as Evans blue or Congo red will quench nonspecific staining and improve contrast.

Fluorescence microscopy is more demanding than ordinary light microscopy, since objects are always much dimmer. A conventional microscope of good quality can be used. There is no need for quartz optics, even though an ultraviolet light source is used. The usual physical arrangement is depicted in Fig. 14-6. A high-pressure lamp emitting ultraviolet and short blue light is needed. The light is filtered by the primary filter to remove light longer than 450 to 500 nm. Heat filters usually are required because of the intensity of the mercury lamp. A front-surfaced mirror diverts the light into the condensor. A darkfield condensor is preferred, because it is easier to see a

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Applications in Confocal Microscopy

Fluorescence Lifetime Imaging Microscopy (FLIM)

Multi-color staining with fluorescent dyes is actively used for observing the distribution of biological materials (such as proteins, lipids, nucleic acids, and ions) in the field of tissue and cell research. The detection technology for fluorescence observation has advanced to a level at which a single fluorescent dye molecule can be detected under the best of circumstances. This section reviews several of the important aspects of fluorescence lifetime imaging microscopy (**FLIM**), a new fluorescence microscopy technology. In addition to multi-color staining, fluorescence lifetime imaging can also be utilized to visualize the factors that affect the fluorescence lifetime properties of the dye molecule, that is, the state of the environment around the molecule.

Wavelength Spectroscopy

Conventional fluorescence microscopy makes use of the color properties of fluorescent dyes, that is, identification is based on differences in fluorescence spectral characteristics between dyes. With this technique, five or six dyes in the wavelength range from ultra violet to near infrared can be used simultaneously under microscopy with no confusion between colors.

Lifetime Spectroscopy

Each fluorescent dye has its own lifetime in the excited state. By detecting differences in lifetime, it is possible to distinguish even dyes having the same fluorescent color as well as to identify autofluorescence. Furthermore, high signal-to-noise images can be obtained by using a probe with very long lifetime compared to that of the fluorescent dyes normally used. For instance, platinum coproporphyrin has a lifetime of millisecond order while the lifetimes of ordinary fluorescent dyes are of nanosecond order. Such relatively long-lived fluorescent dyes will soon be used as probes for DNA detection on chips.

Fluorescence lifetime imaging also makes it possible to obtain information on the molecules while observing a living cell. The factors affecting the fluorescence lifetime include ion intensity, hydrophobic properties, oxygen concentration, molecular binding, and molecular interaction by energy transfer when two proteins approach each other. Lifetime is, however, independent of dye concentration, photobleaching, light scattering and excitation light intensity. Therefore, fluorescence lifetime imaging allows us to perform accurate ion concentration measurement and Fluorescence Resonance Energy Transfer (**FRET**) analysis.

There are two methods of fluorescence lifetime imaging: the time-domain method and the frequency-domain method.

- **Time-domain FLIM** - In some cases of delay after excitation by a pulse laser, the fluorescence image can be obtained by the gate operation of the image intensifier. The lifetime is measured in nanoseconds by a laser with a pulse duration of a few hundred picoseconds and a nanosecond-level shutter because the lifetime of an excitation state is usually 1 to 20 nanoseconds. A high-speed gate image intensifier is commercially available from Hamamatsu Photonics K.K. (Hamamatsu, Japan). The fluorescence

lifetime at each pixel can also be obtained by measuring while varying the delay time until a gate opens. Fluorescence lifetime images are shown in pseudocolor according to their lifetimes.

- **Frequency-domain FLIM** - Fluorescence lifetime is calculated by measuring the phase shift of fluorescence and the reduction in its amplitude using a detector with a gain modulator when the laser used as the excitation light source is modulated (1 to 200 megahertz). The measurement made be taken either by laser scanning or by charge-coupled device (**CCD**).

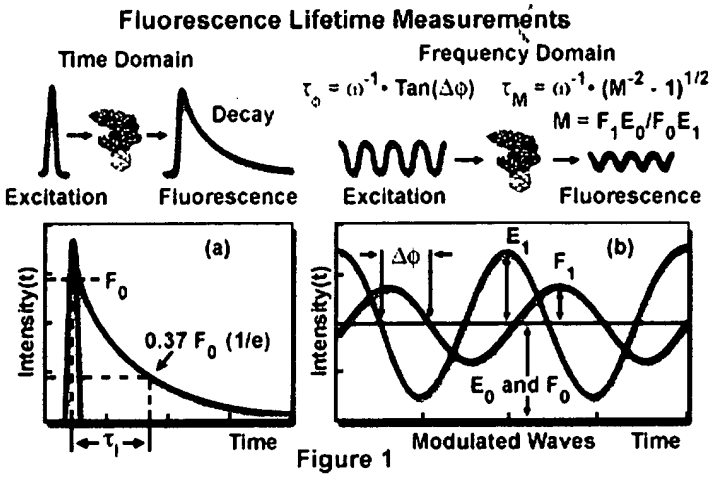


Figure 1

Applications

The environment surrounding the probe is detected based on the fact that the fluorescence lifetime is sensitive to hydrogen ion concentration (**pH**), oxygen, and calcium ion concentrations. The binding or the interaction between molecules can also be measured in combination with FRET.

Calcium Ion Concentration Imaging

When the calcium ion binds to a fluorescent probe such as Fura-2, Fluo-3 or Calcium Green, both the fluorescence lifetime and the fluorescence intensity change. The conventional procedure for ion concentration measurement focuses on the change in intensity. According to the change of the calcium ion concentration, the ratio of dyes between bound and unbound calcium ion changes, and this subsequently leads to a change in the fluorescence lifetime of the measuring spot in the specimen. In addition to the calcium ion probe, this technique is also applicable to the measurement of pH and other ions such as sodium ion and magnesium ion.

Fluorescence Resonance Energy Transfer (FRET)

Research is currently being conducted on FRET by green fluorescent protein (**GFP**) variants (GFP with a different fluorescence color). FRET makes it possible to measure the interactions (association or dissociation) between two proteins that are labeled with a pair of fluorescence dyes. A donor fluorescent dye has shorter excitation/emission wavelengths that provide energy to an acceptor fluorescence dye. The lifetime of the excitation state of the donor dye is variable depending on whether or not the acceptor (the dye receiving the energy) exists. Measurement based on lifetime permits better quantification because it is not necessary to consider the overlap of fluorescence during detection.

Clinical Imaging

As some tissue and cytodiagnostic specimens have strong autofluorescence, the use of probes with long lifetimes (up to milliseconds) has been attempted. Long-lifetime probes are also useful in Fluorescence ***in situ*** hybridization (**FISH**) because the number of colors that can be used simultaneously is limited with this technique. The hydrogen ion concentration in blood, as well as the oxygen and carbon dioxide pressures, have already been measured based on fluorescence lifetime, although such measurements are still not possible under microscopy.

Internet Resources

- **Center for Fluorescence Spectroscopy** - Hosted by Professor Joseph R. Lakowicz at the University of Maryland, this website is an excellent resource for information about fluorescence lifetime imaging and other aspects of fluorescence spectroscopy and microscopy.

- **Kentech Instruments** - Kentech manufactures high voltage solid state pulse generators and optical gated imaging systems for fluorescence lifetime imaging.

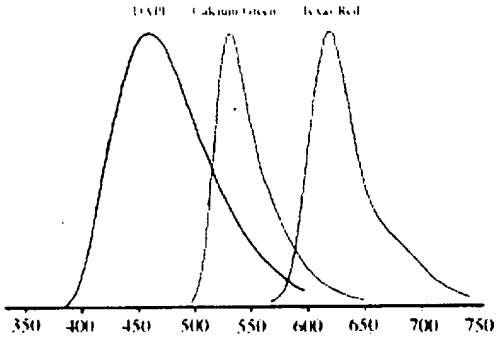
- **Hamamatsu Photonics** - In addition to their excellent lineup of digital camera systems, Hamamatsu also manufactures photomultipliers, avalanche photodiodes, and high-speed gate image intensifiers.

- **PRS BioSciences** - Specializing in biological fluorescence microscopy, PRS BioSciences manufactures an aftermarket time-gated system that can be adapted to many research microscopes.

BACK TO APPLICATIONS IN CONFOCAL MICROSCOPY

Development of a combination spectral/lifetime detector. We are currently developing a combined spectral/lifetime detector that is optimized for low-light level multiphoton imaging. The detector works in photon counting mode and essentially sorts detected photons into spectral and temporal bins. This detector is being developed primarily for the Optical Workstation but will also be used with the high-speed multiphoton imaging system currently under development.

Spectral imaging is the collection and display of the spectral components of a fluorescence image. LOCI is actively engaged in the development of a novel spectral detector that is optimized for multiphoton imaging. The spectral detector will be implemented on the Optical Workstation and the high-speed multiophoton imaging system currently under development.



Benefits of spectral imaging. Most commercial confocal and multiphoton microscopes currently have the ability to collect two or three pre-specified colors simultaneously. However, there is often a need for more complete spectral information to allow the detection of more fluorophores and to facilitate the setting of spectral windows to optimize detection of a specific fluorophore. The main goal of the detection system is to collect the desired signal in the presence of noise (detection noise, system noise, fluorescent background etc.). Background fluorescence from endogenous fluorophores or from another interfering exogenous fluorophores can severely reduce detection &endash; or interpretation -- of the image signal. With multiple labeled samples, the signal from one fluorophore is often much stronger than another and can spill over to an adjacent channel. This problem is exacerbated by fluorophores with extended red tails: DAPI, for example. In these instances it is often better to move the spectral detection windows as far apart as possible to aid discrimination between the two fluorophores being studied rather than choosing spectral windows to give the maximum signal in each channel.

The use of multiple fluorescent labels has long been commonplace in the study of fixed specimens, and is now becoming established for in vivo studies. Not so long ago only three fluorophores were in widespread use (fluorescein, rhodamine and DAPI); now there is a plethora of fluorophores available, each with its own unique spectral characteristics. This has generated a considerable problem for fluorescence microscopists in that many different filter sets are required for double or triple labeled samples. Filter sets use expensive interference filters and dichroic mirrors and are often difficult to interchange. Ideally, filters should be continuously adjustable so that for any particular combination of fluorophores used, an optimal set of band-pass assignments can be selected for each detection channel to minimize signal bleed-through and maximize the signal-to-noise ratio. Perhaps the greatest power of collecting the entire spectrum is this allows fluorophore to be identified and separated computationally (by comparison to reference fluorophore spectra) in the presence of high levels of background.

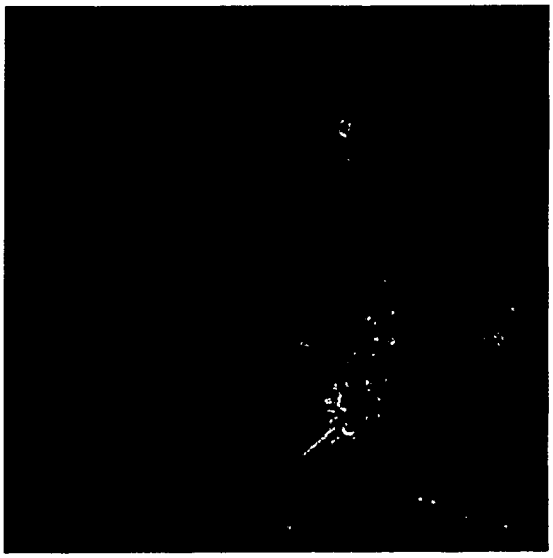
Most biological tissue is autofluorescent. Molecules such as NAD(P)H, elastin, and chlorophyll act as endogenous fluorophores. Often, these endogenous fluorophores can be identified by their characteristic spectra. A spectral imaging system is of considerable use in identifying endogenous fluorophores and specifying spectral windows that would either maximally accept or reject these signals, depending on the application. Additional information may

obtained by comparing spectra obtained at diferent excitation wavelengths.

The use of engineered fluorescent probes as physiological indicators has become a well-established technique. Some probes indicate the presence of a bound ligand by changes in fluorescence intensity (e.g. Calcium Green 1) while others use spectral shifts (e.g. Indo 1). The later are favored because ratio imaging at two different wavelengths may be used to provide measurements that are independent of the concentration of the indicator molecule, measurements that are quantitative. Spectral detection allows an optimum set of spectral windows to be used for ratio imaging.

Fluorescence resonant energy transfer (FRET) is a powerful technique for measuring intermolecular distances in vivo (dos Remedios & Moens, 1995. J. Struct. Biol. 115, 175). This technique also requires custom filter sets that are matched to the donor and acceptor molecule's emission spectra. Ratiometric measurements are used to measure the extent of resonance transfer. FRET is proving to be a valuable technique for the *in vivo* visualization of the docking of a receptor with its ligand, and it is the basis of operation of a new GFP based calcium indicator, Cameleon (Miyawaki et al., 1997 Nature 388, 882-887).

Fluorescence in situ hybridyzation (FISH) is another very significant area where multiple fluorophores and ratiometric techniques are used (Dauwerse et al., 1992 Hum. Mol. Genet. 1, 593). Often the main requirement in this application is to spectrally resolve as many separate fluorescent probes as possible (Schröck et al., 1996 Science 273:494).



Many standard histological preparations are fluorescent. Often the spectra of the fluorophore differs in a tissue specific manner. This property could be an aid to structural identification and thence to diagnoses in pathological specimens. This is an MP image of a 200µm thick specimen of kidney tubules stained with acid fucsin. Spectral windows: top left, 580 to 630nm; top right 500 to 550nm; bottom left, 390 to 485nm; Bottom right, pseudocolor merge The specimen was prepared by Al Kutchera of Midwest Microtech, Inc

The following list summarizes the main advantages of a spectral imaging system over a conventional, filter-based three-channel detector:

- Dynamic identification of auto-fluorescence and optimization of windows for rejection or imaging as required.
- Dynamic optimization of spectral windows for multiple labels.
- Dynamic background subtraction of reference spectra before the image is even displayed
- Identification of spectral shifts of fluorophores in different environments e.g. bodipy ceramide Golgi marker or

acridine orange binding to RNA or DNA.
- Full signal optimization for any given ratiometric indicator.
- Permits fluorophore separation after data collection if full spectral image is taken.
- Permits evaluation of standard histological procedures for MPLSM analysis for identification of tissue-specific spectral shifts of staining.

Fluorescence excited-state lifetime imaging. Time-resolved fluorescence spectroscopy is a well-established technique for studying the emission dynamics of fluorescent molecules i.e. the distribution of times between the electronic excitation of a fluorophore and the radiative decay of the electron from the excited stated producing an emitted photon. The temporal extent of this distribution is referred to as the fluorescence lifetime of the molecule. Lifetime measurements can yield information on the molecular microenvironment of a fluorescent molecule. Factors such as ionic strength, hydrophobicity, oxygen concentration, binding to macromolecules and the proximity of molecules that can deplete the excited state by resonance energy transfer can all modify the lifetime of a fluorophore. Measurements of lifetimes can therefore be used as indicators of these parameters. Furthermore, these measurements are generally absolute, being independent of the concentration of the fluorophore. This can have considerable practical advantages. For example, the intracellular concentrations of a variety of ions can be measured in vivo by fluorescence lifetime techniques (Szmacinski et al., 1994 Methods Enzymol. 240, 723). Many popular, visible wavelength calcium indicators, such as Calcium Green 1, give changes of fluorescence intensity upon binding calcium. The intensity-based calibration of these indicators is difficult and prone to errors. However, many dyes exhibit useful lifetime changes on calcium binding and therefore can be used with lifetime measurements (Lakowicz, et al., 1994 Cell Calcium 15, 7). This gives the considerable advantage that absolute measurements of concentration can be made with no elaborate calibration procedures required. Alternatively, lifetime measurements may be used to calibrate the intensity signals from these indicators when maximum sensitivity is required.

An exciting new development of the field has been the development of the technique of fluorescence lifetime imaging microscopy (Lakowicz et al., 1992 Anal. Biochem 202: 316; Wang et al., 1992. Crit. Rev. Anal. Chem. 23: 369; Gadella et al., 1993. J. Cell Biol. 129, 1543). In this technique lifetimes are measured at each pixel and displayed as contrast. Lifetime imaging systems have been demonstrated using wide-field (Lakowicz et al., 1992 Anal. Biochem 202: 316, confocal (Sanders et al., 1995 Anal. Biochem. 227: 302 and multiphoton (French et al., 1997. J. Microsc. 185: 339) imaging modes. FLIM combines the advantages of lifetime spectroscopy with fluorescence microscopy by revealing the spatial distribution of a fluorescent molecule together with information about its microenvironment. In this way an extra dimension of information is obtained. This extra dimension can be used to discriminate among multiple labels on the basis of lifetime as well as spectra. This would allow more labels to be discriminated simultaneously than by spectra alone in applications where many labels are required such as FISH, for example. There are also promising applications of lifetime imaging in the medical sciences. For example, tumors have been detected in mice sensitized with a hematoporphyrin derivative by lifetime imaging (Cubeddu et al., 1997 Photochem Photobiol 66(2):229).

We are particularly interested in the possibilities that are opened up by multiphoton lifetime imaging of live specimens. In these applications lifetime imaging, in conjunction with spectral imaging should greatly facilitate studies using ion indicator probes and FRET studies of intermolecular distances. For example, a remarkable calcium indicator has recently been described that is a chimeric protein based on two spectrally distinct forms of fluorescent protein (cyan and yellow) and a calmodulin molecule (Miyawaki et al., 1997 Nature 388: 882). Being a naturally fluorescent protein, genetic transformants can be made so that transformed animals will express the indicator in a range of cell types determined by the promoter. The excitation wavelength is chosen to primarily excite the cyan fluorophore. On binding calcium, the calmodulin portion of the molecule changes conformation bringing the two fluorophore regions closer together allowing resonant energy transfer between the cyan and the yellow. This will cause a shift of the emitted spectrum from cyan to yellow. The development of this engineered protein (known as Cameleon) is a remarkable development as it circumvents all the problems associated with loading probes into cells since stable transgenic lines can be used which all express Cameleon. However, one of the problems with Cameleon

is that, although ratiometric methods can be used, the signal change on binding calcium is quite small making this indicator less sensitive than other indicators such as Calcium Green. Lifetime measurements are a sensitive indicator of FRET (Godella et al., 1995. J. Cell Biol. 129, 1543) and in combination with spectral measurements, should provide a more sensitive indication of calcium levels.

Techniques for lifetime imaging. Fluorescent lifetimes can be measured either in the frequency domain or in the temporal domain. Three general strategies have been used to measure fluorescence lifetimes:

- Frequency-domain imaging. In this scheme a high-frequency, modulated light source is used for fluorophore excitation. By the use of a gain-modulated detector, the phase shift and amplitude demodulation of the fluorescence signal is determined. From these data the fluorescent life-time of the probe can be calculated. This scheme is robust and has been extensively used (Wang et al., 1992). However for our purposes it suffers from several drawbacks: the detector is only working at 50% of its maximum efficiency because it is gain modulated, several data sets taken at different excitation modulation frequencies have to be taken in order to separate two or more lifetime components and finally, this scheme does not work well with photon counting techniques which we favor for the reasons described in the section describing the spectral detector.
- Time-domain lifetime imaging with gated detectors. In this scheme a gated micro-channel plate image intensifier is used in conjunction with a CCD imaging camera (e.g. Straub and Hell, 1998. Applied Physics Letters 73:1769). Spectral information is obtained by gating the image intensifier on for a narrow time-window at progressively later intervals after the excitation pulse in a succession of data frame captures. This scheme is probably the simplest way of implementing a life-time imaging system. However, it suffers from two major drawbacks for our application: the method has very poor photon utilization as only one temporal interval is detected at a time. If there are 32 intervals for example, 31/32 of the signal is not utilized and 32 separate frames have to be captured. The second reason this scheme is not appropriate for a multiphoton imaging application is that an imaging (i.e. area) detector is used. This means that the deep sectioning advantage of multiphoton imaging are not fully realized because scattered fluorescence emission photons will give rise to background noise rather than contributing to the signal as can be done with a point-scanning multiphoton system.
- Time-domain lifetime imaging with photon counting. For working at low-light levels, photon-counting detectors have considerable advantages in that they can virtually eliminate noise contributions from electronic amplifiers or electron multiplier noise in a photomultiplier. Also, photon-counting systems provide quantized pulses for every detected photon, allowing the lifetimes to be measured directly using electronic circuitry. Because of the very high speeds necessary to obtain sub-nanosecond temporal resolution., time-to-voltage converters are usually used to measure the interval between the fluorophore excitation pulse and the time of detection of the emitted fluorescent photon. Such schemes have been successfully used in practical photon-counting lifetime detectors (Kelly et al., 1997. Rev. Sci. Instrum 66(6):2279). These schemes are attractive because of their efficient utilization of detected photons. However they suffer from dynamic range problems that arise out of limited counting speeds. Typically, a time-to-voltage converter together with an associated analogue (voltage) to digital converter would have a maximum counting rate of around 1Mhz. Also, with this scheme, only one photon can be measured in the interval between laser pulses. These limitations restrict the use of this technique to low light levels when fairly long exposure times are needed in order to obtain sufficient counts for accurate representation of the decay curve. The comparatively large dead-time of this technique can have more insidious consequences. Immediately after the laser pulse, photons will be emitted at the highest rate and therefore more will be preferentially lost at this time because of the dead-time of the detector. This effect can distort the shape of the decay profile.

Applications of lifetime imaging.

At LOCI we are interested in lifetime measurements as a means of providing another dimension of information from fluorescent probes used in vivo. We find that in most applications where probes are viewed in 4-dimensions *in vivo*,

we would benefit from more or better information. Specifically we anticipate that the combined MP spectral and lifetime imaging system will provide the following benefits to our collaborators:

- More accuracy in ratiometric probe measurements. This will be achieved by choosing optimal spectral and lifetime parameters that give the maximum shift with the probe target signal (e.g. Ca++ concentration).
- Lifetime imaging will enable some popular, non-ratiometric probes, such as calcium green, to be used in a way that is concentration independent, thereby facilitating calibration of readings.
- In conjunction with spectral imaging, lifetime imaging will improve the rejection of background fluorescence from endogenous fluorophores by the specification of optimum spectral and temporal windows. This is becoming an increasing important requirement for detecting low levels of GFP probe amid a background of autofluorescence.
- Lifetime measurements add extra information that can be used in conjunction with spectral measurements for fluorophore identification. This will be useful when there is significant spectral overlap between probes. This technique should allow the use of a greater number of probes simultaneously, such as combinations of GFP variants.
- Increasingly cell biological studies are using FRET for studies of protein/protein interactions or physiological parameters in vivo. There is usually a striking change in the lifetime of the donor and acceptor fluorophores undergoing a FRET interaction. Lifetime imaging may well prove superior to spectral ratio imaging for measuring FRET interactions. A combination of lifetime and spectral imaging will probably be better still.

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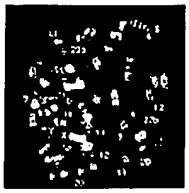
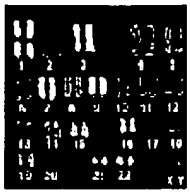
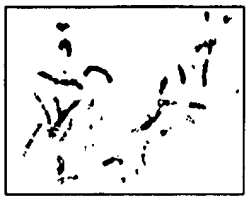


New Methods for Karyotyping



Introduction: The Spectral Karyotype

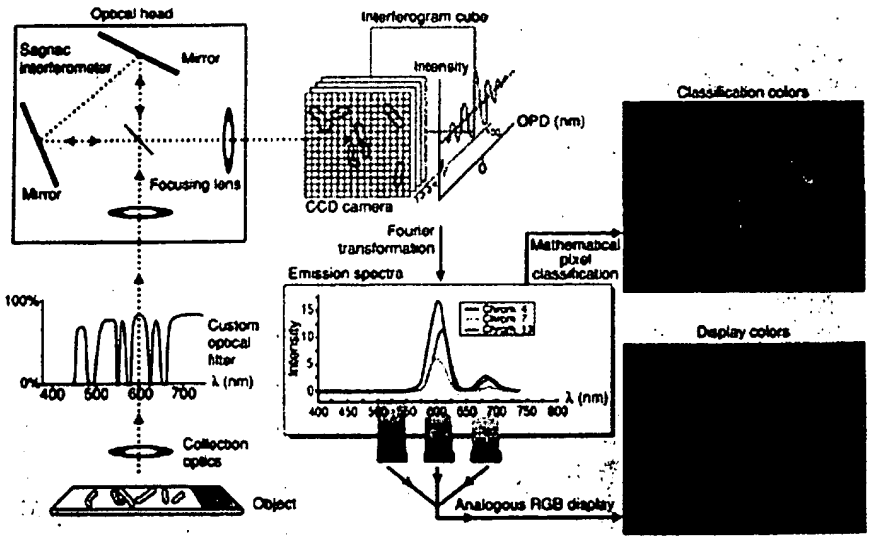
A scientific group from the National Center for Human Genome Research in Bethesda, MD has recently published a modification to traditional karyotyping that permits rapid identification of chromosomal alterations. These findings are of import because the ability to detect altered chromosomes is increasingly important for pre- and postnatal diagnostics and in cancer and other diseases.



The traditional process for karyotyping involves adding a dye to metaphasic chromosomes. Different dyes that affect different areas of the chomosomes are used for a range of identification purposes. One common dye used is Giemsa; That process is known as G-banding (see the G-banded chromosomes in the image to the left). This dye is effective because it markedly stains the bands on a chromosome; Each chromosome can then be identified by its banding pattern, but the resuls is similar overall gray values for each chromosome.

The new karyotyping methods introduced by Schrock *et al* use fluorescent dyes that bind to specific regions of chromosomes. By using a series of specific probes each with varying amounts of the dyes, different pairs of chromosomes have unique spectral characteristics. A unique feature of the technology is the use of an interferometer similar to ones used by astronomers for measuring light spectra emitted by stars. Slight variations in color, undetectable by the human eye, are detected by a computer program that then reassigns an easy-to-distinguish color to each pair of chromosomes. The result is a digital image rather than film, in full color. Pairing of the chromosomes is simpler because homologous pairs are the same color, and abberrations and cross-overs are more easily recognizable. In additional, the spectral karyotype has been used to detect translocations not recognizable by traditional banding analysis.

A summary of the spectal karyotyping methods.



The paper:

Multicolor Spectral Karyotyping of Human Chromosomes

SCIENCE 26 Jul 1996; 273 (5274):494 (in Reports)

E. Schröck, S. du Manoir, T. Veldman, B. Schoell, J. Wienberg, M. A. Ferguson-Smith, Y. Ning, D. H. Ledbetter, I. Bar-Am, D. Soenksen, Y. Garini, T. Ried

Editorial:

CYTOGENETICS: New Methods for Expanding the Chromosomal Paint Kit

SCIENCE 26 Jul 1996; 273 (5274):430 (in Research News)

METHODS ▶

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Tuesday, August 13, 1996
Contact the Development Team